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HISTOLOGY AND HISTOCHEMISTRY OF THE OVARY AND OOGENESIS IN *BALANUS AMPHITRITE* L. AND *B. EBURNEUS* GOULD (CIRRIPEDIA, CRUSTACEA)

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The oogenesis and microanatomy of the cirriped ovary are only partly described in earlier literature (Darwin, 1851, 1854; Krohn, 1859; Gruvel, 1905). More recently, ovarian maturation has been studied by visual observation of size, color and texture of the ovary mass (Crisp, 1954; Crisp and Davies, 1955; Patel and Crisp, 1960; Barnes, 1963; Barnes and Barnes, 1967) and by measurements of egg size (Crisp, 1954; Barnes and Stones, 1973). Utinomi (1960) and Turquier (1972) described briefly the ovary structure and oogenesis in acrothoracic cirripeds, and a brief description was made of cultured and uncultured ovarian tissue of the balanids (*Balanus amphitrite* and *B. eburneus*) by Fyhn and Costlow (1975). However, a more detailed microscopical examination of cirriped ovaries based upon histochemical techniques has not been available. The aim of the present study is to give data on the histology and histochemistry of the ovary and oogenesis of cirripeds represented by the two thoracic barnacles (*Balanus amphitrite* L. and *B. eburneus* Gould).

Materials and Methods

Specimens of the acorn barnacle *Balanus amphitrite* (basal diameter 10 to 15 mm) were collected in October at the dock of Duke University Marine Laboratory, Beaufort, North Carolina. Specimens of *B. eburneus* (basal diameter 15 to 25 mm) were collected at the same place in August. The animals were fixed immediately upon collection. In addition, specimens of *B. amphitrite* collected during the months of April to June were fixed after being maintained for 10 to 14 days without food in aquaria at a salinity of 30‰ and 23 ± 1° C. The water was changed weekly, and the animals were then cleaned by light brushing. Prior to fixation some of the animals were molt staged according to the method of Davis, Fyhn and Fyhn (1973) using the rami of the sixth pair of cirri. The body and opercular valves were removed from the shell after cutting the opercular membrane. The shell with mantle tissue and ovarioles was fixed for two hours in acetic alcohol (3 parts of ethanol: 1 part of acetic acid). After washing in 100% ethanol, the mantle tissue was dissected out, embedded in Paraplast (Fisher Scientific) and serial sectioned at 8 μ. For general orientation, the Mallory-Heidenhain Azan stain was used (Koneff, 1938). For histochemical studies the following methods were used: (for proteins) Mercury-bromphenol blue after Bonhag (Pearse, 1968), Million reaction Baker modification (Pearse, 1968), Thioglycollate-ferric ferricyanide (Adams, 1956), DDD reaction (Barnett and

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Seligman, 1952) with alkylation for control, Thioglycollate/DDD reaction (Barnett and Seligman, 1952); (for carbohydrates) Toluidine blue O (Pearse, 1968; Lillie, 1929) with RNAse treatment for control, Azure A (Kramer and Windrum, 1955) with RNAse treatment for control, Mucihematein (Laskey, 1950) with RNAse treatment for control, Alcian blue (Steedman, 1950) with RNAse treatment for control, Periodic acid Schiff's reaction with acetylation and deacetylation for control (Casselman, 1962; Lillie, 1965), for DNA—Feulgen reaction (Feulgen and Rossenbeck, 1924), for DNA and RNA—Methyl-green-pyronin Y (Kurnick, 1955) with RNAse treatment for control; and (for lipids) Sudan black B (Pearse, 1968) with pyridine extraction for control.

Photomicroscopy was made with a Zeiss photomicroscope II and the size measurements were made with a *camera lucida* (magnification up to 813×).

Results

The ovaries of *B. amphitrite* and *B. eburneus* consist of branched tubules (ovarioles) located in the connective tissue between the mantle cavity and the basal membrane. Four types of cells can be distinguished in the ovarioles (Fig. 1): cells of the ovariole membrane, oogonia, previtellogenetic oocytes in various stages of development (immature oocytes), and oocytes with discernible yolk droplets (mature oocytes). Interstitial or follicle cells were not observed around the oocytes. The cells of the ovariole membrane make up a continuous lining (ca. 0.25 to 0.50 μ thick) of the ovarioles. The cells are flattened with a cytoplasm staining light blue with Azan and showing weakly positive reactions in the tests for RNA and negative reactions in the other histochemical tests applied. Mitotic configurations were not observed in these cells. The oogonia are closely

Table I

Histochemical reactions of the cytoplasm of immature oocytes and of yolk droplets of mature oocytes in Balanus amphitrite *and* B. eburneus.

Test	Immature oocytes	Yolk droplets
Azan	yellow	red
Mercury-bromphenol blue	−	+++
Million reaction	−	+
Thioglycollate ferric ferricyanide	−	+++
DDD/control	−/−	++/−
Thioglycollic reduction, DDD	−	+++
Toluidine blue O/control	dark blue/−	−/−
Azure A/control	dark blue/−	−/−
Mucihematein/control	+/−	−/−
Alcian blue/control	bluegreen/−	−/−
Periodic acid Schiff's/control	++/−	+/−
Feulgen reaction	−	−
Methyl-green-pyronin Y/control	+++/−	−/−
Sudan black B/control	+/−	−/−

+++ Strongly positive reaction.
++ Moderately positive reaction.
+ Weakly positive reaction.
− Negative reaction.

packed cells making up a continuous string of cells in the ovariole. The cells are rounded with a diameter of about 2 μ and were frequently seen in mitosis. Pycnotic nuclei were not apparent in the oogonia. The cytoplasm of the oogonia showed positive reactions in the tests for RNA (Fig. 2) and negative reactions for carbohydrates and proteins. Immature oocytes (5 to 45 μ) have a large nucleus with one nucleolus (Fig. 1). Chromosome structures or premeiotic configurations (Raven, 1961) were not clearly seen. The histochemical reactions of the cytoplasm of immature oocytes are listed in Table I. The cytoplasm showed negative reactions in the tests for proteins (Fig. 3) and strongly positive reactions for RNA (methyl-green-pyronin Y, Fig. 2). The basophilic reactions observed by Toluidine blue O, Azure A and Alcian blue were absent after treatment with RNAse, indicating that the reactions with these stains might be due to RNA. No true metachromasia was observed. In the largest immature oocytes (diameter larger than 40 μ), the cytoplasm was more coarsely granulated and showed weaker basophilia than that of smaller oocytes. The cytoplasm showed a moderately positive reaction in the PAS test, which disappeared by acetylation and reappeared by deacetylation with KOH. This indicates that the positive reaction might be caused by 1, 2 glycol groups of carbohydrates. The cytoplasm showed a positive reaction with Sudan black B, and negative reaction after treatment with pyridine. The test was applied to fixed material, however, and gives positive reaction for large amounts of sudanophilic lipids only, since most of the lipids are dissolved by the fixative. Mature oocytes are approximately spherical with a diameter of 45 to 70 μ. The nucleus (10 to 12 μ) has one nucleolus. Meiotic chromosomes were not observed. The cytoplasm is filled with spherical yolk droplets with a diameter of 3 to 5 μ in *B. amphitrite* (Fig. 1) and 4 to 6 μ in *B. eburneus* (Fig. 3). In most oocytes the droplets were evenly distributed throughout the cytoplasm. In some oocytes, however, the periphery of the cytoplasm was free of droplets and the droplets were significantly smaller (1 to 3 μ in *B. amphitrite*) and less clear in appearance. Histochemical reactions of the yolk droplets of mature oocytes are shown in Table I. The droplets showed positive reactions in the protein tests (Fig. 3) and negative reactions in the tests for RNA (Fig. 2), acid mucopolysaccharides and mucin. The droplets showed weakly positive reactions for 1,2 glycol groups. The cytoplasm between the yolk droplets showed positive reactions in the tests for RNA (Fig. 2), lipids, and 1,2 glycol groups, and negative reactions in the tests for proteins (Fig. 3).

The ovarioles showed changes in structure and cellular composition. Small ovariole buds with a diameter of 20 to 25 μ in *B. amphitrite* (Fig. 7) and 20 to 30 μ in *B. eburneus* (Fig. 4) occurred along ovarian ducts in most of the animals. The ovariole membrane in the bud has a thickness of 1 to 1.2 μ in *B. amphitrite* and ca. 1.5 to 2 μ in *B. eburneus*. A high density of cell nuclei is observed in the membrane. In the lumen of the buds some cells can be seen, as well as amorphous material (Fig. 4). The latter is dark brown to black regardless of the staining technique applied. The ovariole buds seem to increase in length and width, the ovariole membrane becomes thinner, and its cell nuclei become more dispersed. No mitoses were observed in these nuclei. In slightly elongated ovarioles, a layer of columnar cells with apical nuclei has been organized beneath the ovariole membrane (Fig. 5). In more elongated ovarioles oogonia are found in the center

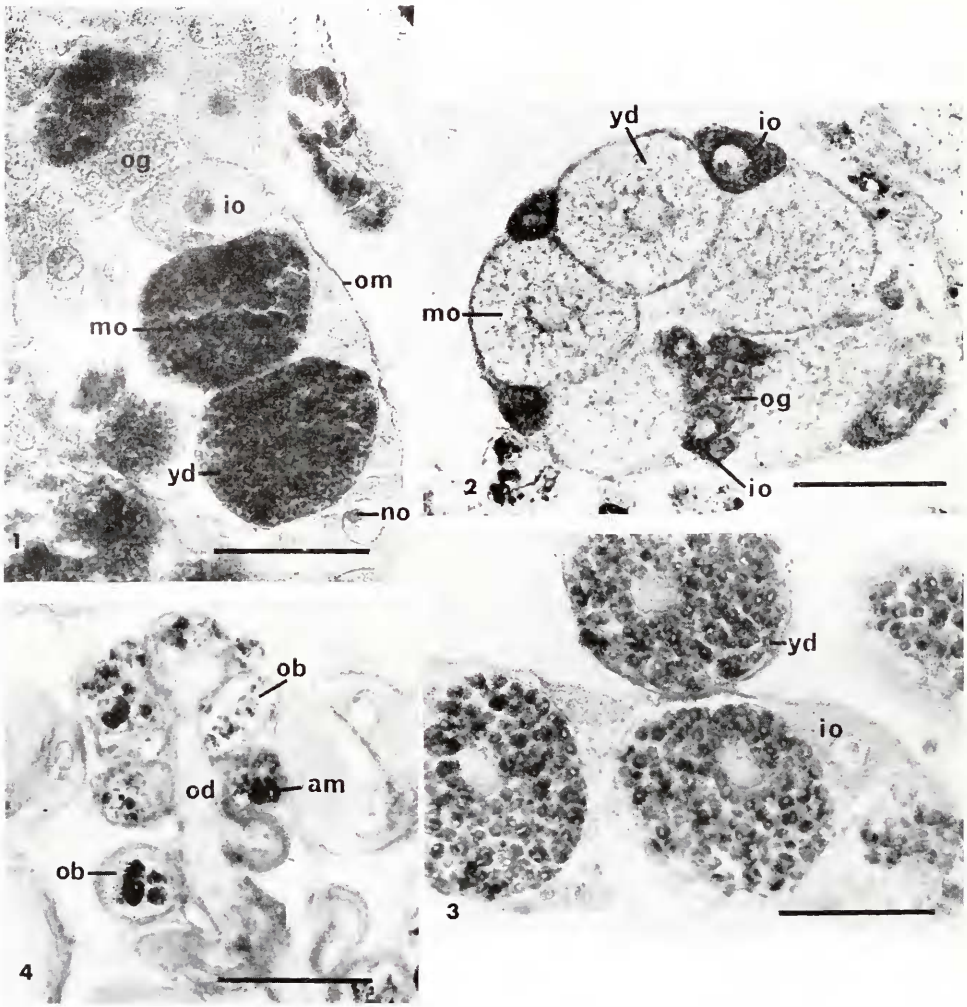

FIGURE 1. Ovariole of *B. amphitrite* with oogonia (og), immature oocytes (io) with nucleolus (no), mature oocytes (mo) with yolk droplets (yd) and ovariole membrane (om) (Azan stain, scale 50 μ).

FIGURE 2. Ovariole of *B. amphitrite* with RNA in the cytoplasm of oogonia (og), immature oocytes (io), and between the yolk droplets (yd) in mature oocytes (mo) (methyl-green pyronin Y, scale 50 μ).

FIGURE 3. Ovariole of *B. eburneus* showing SH- and SS-positive yolk droplets (yd) and negative cytoplasm of immaure oocytes (io) (thioglycollic reduction followed by DDD reaction, scale 50 μ).

FIGURE 4. Ovariole buds (ob) with amorphous material (am) of *B. eburneus* along an ovarian duct (od) (Azan stain, scale 50 μ).

of the ovariole which has become a compact structure. The oogonia are observed in frequent mitoses. In even more elongated ovarioles, small immature oocytes are present in the periphery with the string of oogonia in the center (Fig. 6). As

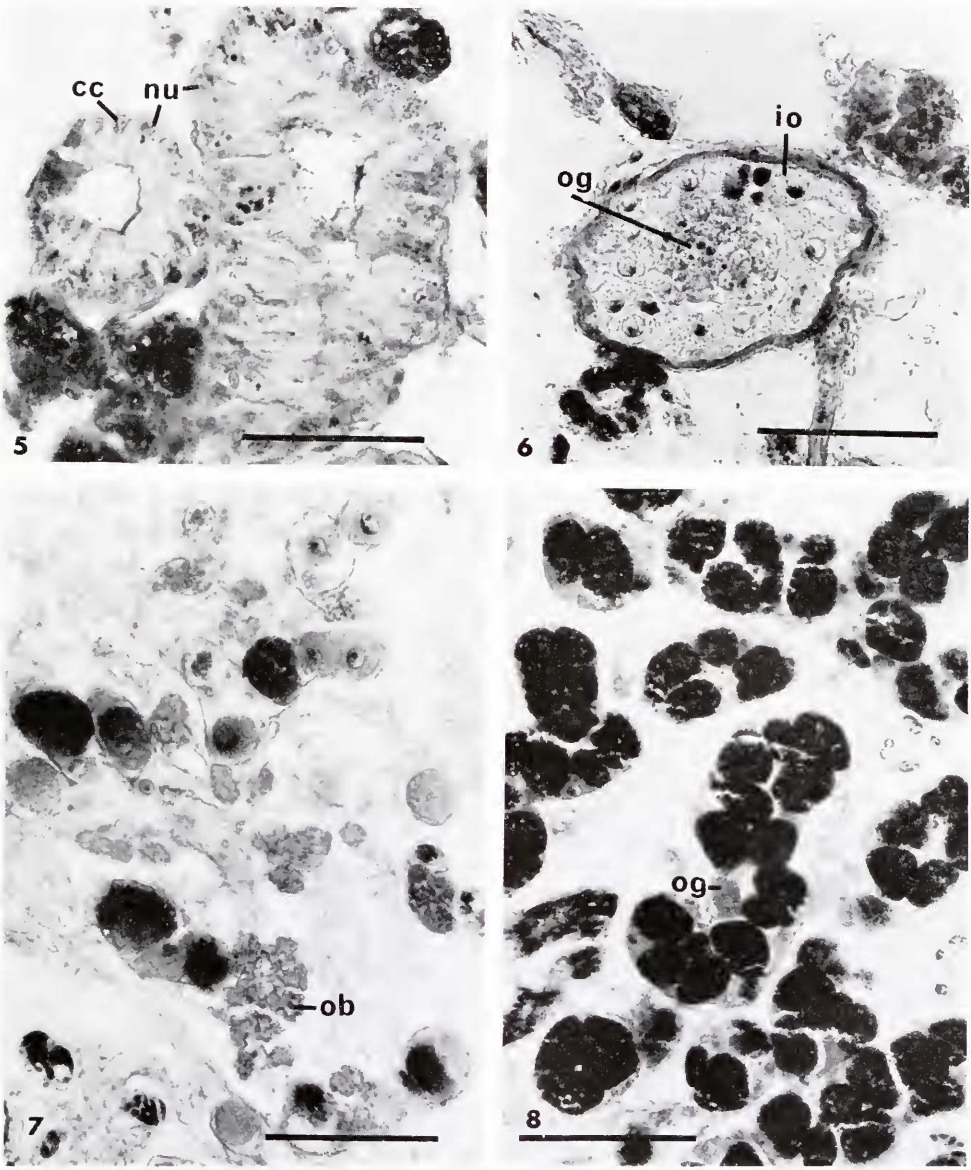

FIGURE 5. Ovarioles of *B. cburneus* with columnar cell (cc) having apical nuclei (nu) (Toluidine blue O, scale 50 μ).

FIGURE 6. Ovarioles of *B. cburneus* with oogonia in the center (og) and immature oocytes (io) in the periphery (Azan stain, scale 50 μ).

FIGURE 7. Ovarioles of *B. amphitrite* with ovariole buds (ob) and oocytes in various stages of development (Azan stain, scale 150 μ).

FIGURE 8. Elongated ovarioles of *B. amphitrite* with oogonia (og) and oocytes in various stages of development (Azan stain, scale 50 μ).

Table II

Ratio of mature to immature oocytes (total number of oocytes in parenthesis) and diameters (mean ± s.e. of the ten largest oocytes measured in each animal) of mature oocytes in ovarioles of Balanus amphitrite *in various stages of the intermolt cycle.*

Molt stage	Ratio of mature to immature oocytes (>20 μ)	Diameter of mature oocytes (μ)
B_1	0.35 (70)	48 ± 1.9
B_2 (early)	0.32 (111)	48 ± 1.1
B_2 (late)	0.76 (187)	55 ± 0.7
C	0.61 (135)	50 ± 1.6
D_1	0.85 (240)	68 ± 0.9
D_1	0.89 (66)	79 ± 1.5
D_2	0.74 (184)	58 ± 0.8
D_2	1.16 (261)	68 ± 2.0

the ovariole becomes further elongated, larger immature oocytes (more than 40 μ in diameter) and mature oocytes appear (Fig. 7). The number of mature oocytes increases with additional elongation of the ovariole (Fig. 8). The ovariole membrane is now 0.3 to 0.5 μ in thickness. The ovarioles are at this stage too long and undulated to be individually traced in serial sections. In transverse section the string of oogonia does not have a different location in the ovariole. Mitoses may be seen in the oogonia, and small immature oocytes (less than 20 μ) are located adjacent to the oogonia (Fig. 2). Immature oocytes of 20 to 30 μ are common in these ovarioles. There are no indications as to a nurse cell-function of these cells, and immature or mature oocytes did not appear in clusters.

Ovariole development and oogenesis were studied in relation to the intermolt cycle in eight specimens of *B. amphitrite* (molt stage B_1, B_2, C, D_1, and D_2) fixed immediately upon collection. Ovariole buds as well as elongated ovarioles with oogonia in mitoses, immature oocytes and mature oocytes were found in all stages of the intermolt cycle. The ratio of mature oocytes to immature oocytes larger than 20 μ in diameter was measured in sections stained with Toluidine blue O. This ratio increased from post- to proecdysial stages (Table II). The diameter (*i.e.*, the average of the longest and shortest diameter) of 20 to 50 mature oocytes of each animal was measured in sections stained with Azan. The mean of the ten largest oocytes in each animal showed a significant increase from postecdysis to proecdysis (Table II).

In animals maintained without food in aquaria for 10 to 40 days before fixation, immature oocytes larger than 20 μ in diameter were degenerating, and the degeneration became more evident with increasing time in aquaria. Smaller oocytes and oogonia seemed normal in most animals. The diameters of the mature oocytes were not significantly altered from the diameters in animals fixed immediately upon collection. The size and number of yolk droplets were normal.

Discussion

A clear distinction between a germarium and a vitellarium was not found in the ovaries of *B. amphitrite* and *B. eburneus*. The germarium has no strictly

defined localization but may be found in the periphery as well as in the center of the ovariole. The vitellarium consists of oocytes only, with apparently no accessory cells. Gruvel (1905) and Krüger (1940) claim that most of the oocytes in the ovary of pedunculate barnacles degenerate and function as a nutritional source for the growing oocytes. In branchiopods and ostracods, nurse cells are associated with the growing oocytes (Raven, 1961); and in decapods, the oocytes are surrounded by follicle cells (Herrick, 1911; Beams and Kessel, 1963). In anostracans (Linder, 1959) and isopods (Balesdent, 1965), the cells of the ovariole membrane have been described as follicular. In the present study of the barnacle ovary, no indications as to a nurse-cell function or a degeneration of immature oocytes were found. Follicle cells around the oocytes were not observed, and there was no evidence of a follicular function of the cells of the ovariole membrane. Most likely, therefore, the barnacle oocyte takes up nutrients through the cell membrane directly from its surroundings complying with the definition of an autonomous egg formation (Nørrevang, 1968).

The histochemical reactions of the developing oocytes and other ovarian cells in *B. amphitrite* and *B. enurneus* are mostly in agreement with descriptions of other crustaceans (Raven, 1961; Linder, 1959; Fautrez-Firlefijn, 1957). The formation of discernible proteid yolk droplets in the oocytes may be a rapid process, since oocytes containing only a few droplets were never observed. The periphery of the oocytes seems to be the last part to acquire yolk droplets. This is consistent with the finding in the acrothoracic cirriped *Trypetesa* that yolk droplets first appear in the perinuclear zone (Turquier, 1972). The sudden appearance of the yolk droplets in the barnacle oocytes does not necessarily reflect an equally rapid synthesis of yolk substances. The formation of yolk droplets may take place by a condensation process when yolk substances dispersed in the cytoplasm have reached a certain density.

The development of the ovary mass in cirripeds has macroscopically been shown to depend upon the food supply of the animal (Patel and Crisp, 1960; Crisp and Davies, 1955; Barnes and Barnes, 1967). In the present study, barnacles with well-developed ovaries were maintained for 10 to 40 days without food. During this starvation, the proteid yolk was maintained, while large and medium sized immature oocytes degenerated. This agrees with findings on barnacle ovary tissue maintained *in vitro* where proteid yolk was resistant to degeneration during insufficient nutritional supply, while previtellogenetic development was not maintained (Fyhn and Costlow, 1975).

During the breeding season, *B. amphitrite* has subsequent broods, one following closely after the other. In such species although the fertilization may take place at any time during an intermolt cycle, it most frequently occurs soon after ecdysis (Patel and Crisp, 1961). In the present study, mitotic activity in the oogonia and small oocytes adjacent to the oogonia was found in *B. amphitrite* of various stages of the intermolt cycle. This should indicate a continuous production of new oocytes throughout the intermolt cycle. However, an increase in the ratio of mature to immature oocytes from postecdysis to proecdysis was found, and the maximum size of mature oocytes showed an increase during the intermolt cycle. This may imply that vitellogenesis is initiated predominantly in postecdysis and is completed during one intermolt cycle.

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Summary

Four cell types can be distinguished in the ovarioles of *Balanus amphitrite* and *B. eburneus:* cells in the ovariole membrane, oogonia, previtellogenetic oocytes in various stages of development, and oocytes filled with proteid yolk droplets. The germarium does not have a strictly defined localization in the ovariole. The vitellarium consists of oocytes only, with apparently no accessory cells. An autonomous egg formation seems probable. The histochemistry of the ovarian cells is described. The formation of discernible proteid yolk droplets appears to be a rapid process. The development from ovariole buds to elongated ovarioles is described. There is a continuous production of new oocytes throughout the intermolt cycle. Vitellogenesis seems to be initiated predominantly in postecydsis and is completed during one intermolt cycle.

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